Wellesley College
Wellesley College Digital Scholarship and Archive

Honors Thesis Collection

2013

Determining the Role of the Hedgehog Signaling Pathway during Limb Regeneration in the Red Flour Beetle, Tribolium castaneum

Carla Mayela Villarreal
cvillarr@wellesley.edu

Follow this and additional works at: https://repository.wellesley.edu/thesiscollection

Recommended Citation
Villarreal, Carla Mayela, "Determining the Role of the Hedgehog Signaling Pathway during Limb Regeneration in the Red Flour Beetle, Tribolium castaneum" (2013). *Honors Thesis Collection*. 109.
https://repository.wellesley.edu/thesiscollection/109

This Dissertation/Thesis is brought to you for free and open access by Wellesley College Digital Scholarship and Archive. It has been accepted for inclusion in Honors Thesis Collection by an authorized administrator of Wellesley College Digital Scholarship and Archive. For more information, please contact ir@wellesley.edu.

Determining the Role of Hedgehog Signaling Pathway during Limb Regeneration in the Red Flour Beetle, *Tribolium Castaneum*

Carla Mayela Villarreal

Advisor: Dr. Yuichiro Suzuki

Department of Biological Sciences

Submitted in Partial Fulfillment of the Prerequisite for Honors in Biological Sciences

Wellesley College

April 2013

© 2013 by Carla Mayela Villarreal

ACKNOWLEDGEMENTS

Foremost, I would like to express my sincere gratitude and appreciation to my thesis advisor Professor Yuichiro Suzuki for his continuous and unyielding support throughout my research career at Wellesley College. His passion and excitement for developmental biology and scientific inquiry allowed me to develop my own drive for research. Professor Suzuki believed in my capabilities as a young researcher from the very beginning, and I have to acknowledge that without his support and guidance I wouldn't have attained this great honor. My scientific accomplishments, especially the development and writing of this thesis, are due to the exceptional mentoring and encouragement from Professor Suzuki. Thank you for everything you have done for me!

I would also like to give special thanks to my committee members Professor Kimberly O'Donnell and Professor Melissa Beers for all their help, guidance and encouragement throughout the process. My experience as a thesis student has been incomparable due to your continual interest and excitement for my research.

My sincere gratitude also goes to my honors visitor Professor David Haines who has been such an influential individual throughout my college life. Even from halfway across the world, you are always wiling to talk to me and offer me your most genuine advice. Thank you!

Thank you Suzuki Lab for encouraging me and showing your utmost interest and care for my research and me.

I have an amazing network of family and friends who continue to encourage me in all of my endeavors. Specifically, I would like to thank my mom, dad, and my siblings for their immense love and support. And my fiancé, Francisco, thank you for always being there for me!

TABLE OF CONTENTS

LIST OF FIGURES AND TABLES

ABSTRACT

Despite studies performed on many species, including amphibians and insects, the mechanisms regulating limb regeneration remain poorly understood. Hedgehog (Hh) is a major signaling pathway found in most, if not all, animal species, including humans. In vertebrates, Hh signaling has been shown to play key roles during limb regeneration. To determine whether Hedgehog might also play a role during limb regeneration in *Tribolium Castaneum*, Hh signaling was silenced through RNA interference. In normal development, silencing of Hh signaling resulted in alteration of limb morphology. Disruption of Hh signaling during regeneration led to the impairment of blastema growth and subsequent adult limb morphogenesis during metamorphosis. Furthermore, cellular proliferation in the blastema diminished with the silencing of Hh. Our findings indicate that Hh signaling is necessary for regeneration in beetles similar to vertebrates and have broader implications for our understanding of the developmental and evolutionary origins of set-aside cells.

INTRODUCTION

The ability of organisms to regenerate lost body parts is one of the most intriguing phenomena in biology due to its complexity and widespread occurrence across various phyla. Regeneration, the replacement of damaged or missing tissues and structures, has been observed in flatworms (e.g. planarians), echinoderms (e.g. starfish), chordates (e.g. salamanders) and arthropods (e.g. insects). Because many animals in different phyla are capable of regenerating some or all body parts, the ability to regenerate may be an evolutionarily conserved character. Thus, it has been proposed that there may have been a common ancestor for all regenerating species at the base of the metazoan tree (Bely & Nyberg, 2009). Regeneration has been subdivided into several categories. Physiological regeneration refers to the regeneration of a particular structure or tissue that occurs repeatedly throughout an organism's life (Seifert *et al*., 2012). Examples in metazoan species include replacement of blood cells, arthropod exoskeleton replacement (molting), epidermis and gut lining replacement and cervid antler regrowth. In contrast, reparative regeneration occurs less frequently and is usually induced by some form of injury causing the damage or loss of a body part (Seifert *et al*., 2012). Organisms that undergo reparative regeneration include lizards, fish, crustaceans and arthropods. Reparative regeneration can occur at multiple hierarchical levels including cellular, tissue, internal organ, structure and whole body regeneration (Bely & Nyberg, 2009). For example, cnidarians and flatworms can generate an entire individual from a small body fragment, while lizards can only replace certain structures, such as a tail but not a head or a limb. Lastly, the processes by which regeneration proceeds in these organisms are categorized as either morphallaxis or epimorphosis. Morphallaxis refers to regeneration of body parts caused by a reorganization of preexisting cells at the wound site (Carlson, 2007; Seifert *et al*., 2012). Epimorphosis, conversely, describes regeneration resulting from the formation of new cells recruited to the wound site (Seifert *et al*.,

2012). In this study, we will focus on elucidating the mechanisms underlying body structure reparative regeneration.

Process of limb regeneration in vertebrates

Many recent studies are focused on identifying the mechanisms controlling appendage regeneration. The basic steps of epimorphic regeneration are initial wound healing, subsequent blastema formation followed by blastema proliferation and eventual re-patterning (Endo *et al*., 2004). At the most fundamental level, the regeneration of appendages amongst various metazoans proceeds through initial formation of a growth zone at the site of injury or plane of amputation (Brockes, 1997; Endo *et al*., 2004). This process usually begins with the rapid migration of epithelial cells to the wound surface, creating a seal that closes the injury site (Brockes, 1997; Tanaka, 2003). The creation of a wound epidermis is the first observable event in regeneration, but the most crucial step in regeneration arises in the cells lying beneath the wound surface. In urodeles and salamanders, cells present in underlying tissues of the wound epidermis reenter the cell cycle and dedifferentiate, losing their differentiated character. This zone of dedifferentiated cells, progenitor cells and fibroblasts near the site of injury is known as the blastema (Tanaka, 2003). Blastema cells proliferate to produce a mass of cells which eventually exit the cell cycle and become differentiated into essential cells of the limb, such as dermis, bone, connective tissue, and muscle. Interactions between blastema cells and the wound epidermis closely resemble progressions seen during development and are necessary for continued growth of the blastema (Tanaka, 2003). Astonishingly, the blastema will only give rise to structures that are distal to its origin; e.g. a wrist blastema will only give rise to a hand, while a shoulder blastema will produce an entire arm (Brockes, 1997; Nacu & Tanaka, 2011). This characteristic illustrates the importance of the proximodistal (P/D) axis in regeneration as

well as the role that the blastema plays for regeneration across different metazoan species. Many experimental models have been created to explain how patterning and limb positioning are determined in the blastema. Although the exact answer is still unclear, all models imply that the blastema is an autonomous self-patterning unit capable of directing cell fate and growth at very early stages of regeneration (Tanaka, 2003). Once early patterning events are complete, limb morphogenesis and growth proceed to restore an appendage of mature size, ending the process of regeneration. This multistep process continues to fascinate and perplex scientists today; how do tissue repair mechanisms reactivate embryonic programs to attain proper pattern formation and morphogenesis in regenerating body parts? What factors govern the regeneration process and are these factors conserved between organisms? One factor that seems to be at the center of limb regeneration is the presence of a blastema in regenerating tissues (Tanaka, 2003). Without the blastema, tissues are incapable of regeneration, and lack of blastema formation is one of the main reasons why mammals cannot regenerate. Therefore, in order to induce regeneration in other organisms, scientists must first define exactly what a blastema is and determine how to promote blastema development at the site of injury.

Blastema development and regulation

In general, the blastema has been thought to be made up of epidermal and mesodermal tissues which interact to continue the overall development and proliferation of blastema cells. The cellular makeup of blastemas has been long debated, however recent studies suggest that dedifferentiated mature cells with restricted potential as opposed to undifferentiated stem cells make up a large portion of what is known as the blastema (Tanaka, 2003; Kragl *et al*., 2009).

If blastemas are formed from dedifferentiated cells, then one might wonder how these cells know what to become next. Do blastema cells lose previous identity information or do they give rise to other cells similar to their previous existence? Studies involving lineage tracing and GFP tracking revealed that blastema cells can traverse lineage boundaries during the course of regeneration (Tanaka, 2003). However, many cells appear to maintain a memory of their previous identity and only a certain subset become somewhat plastic (Kragl *et al*., 2009).

Several key factors have been shown to be essential for blastema formation and proliferation. Common signaling pathways such as FGF, Hh, and Wnt, known to be important during normal development have also been linked with important processes during regeneration (Singh *et al*., 2012). Recent studies conducted in amphibians have reported the importance of Hh signaling for A/P patterning, blastema formation and cellular expansion during limb regeneration (Singh *et al*., 2012). Vertebrate Hh genes are classified into three categories including sonic (Shh), indian (Ihh), and desert (Dhh). However Shh seems to be the only class required for proper development and regeneration in vertebrates (Stark *et al*., 1998; Avaron *et al*., 2000). In zebrafish, Hh signaling is reactivated following adult fin amputation and seems to be important for blastema formation and maintenance as well as for eventual fin ray patterning during regeneration (Avaron *et al*., 2000). Inhibiting Hh signaling causes the eventual arrest of fin regeneration by inhibiting blastema cell proliferation in the epidermis and mesenchyme (Avaron *et al*., 2000). Given its requirement for stem cell proliferation and maintenance (Martinez-Agosto *et al*., 2007), Hh signaling is a particularly interesting signaling pathway to study in other organisms that can regenerate.

Regeneration in invertebrates

Many similarities exist between regeneration in vertebrates, such as salamanders, and regeneration in invertebrates, such as crickets. For example in both vertebrates and insects, gene clusters such as HOX complexes are organized in a physical order corresponding to the A/P axis of the developing organism (Gaunt *et al*., 1988; Duboule and Dollé, 1989; Graham *et al*., 1989). Much like patterning in vertebrates, patterning in insects requires a distinct set of genes and signaling molecules able to receive and process important locational cues. Developing insect legs have been proposed to have positional information specified along the length of the leg which divides it into anterior and posterior compartments as well as around the leg circumference (French, 1982; Simon & Tabin, 1993). The ability to precisely regenerate the structures that have been amputated depends on a 'positional memory' of the cells at the amputation plane (Wolpert, 1969). After amputation, healing confronts epidermal cells with different positional values which consequently stimulate growth and regeneration of more distal tissues. Hemimetabolous insects, like cockroaches and crickets, undergo adult-like leg development directly during embryogenesis from limb buds, similar to development in vertebrates which also stem from limb bud growth (Simon & Tabin, 1993; Nakamura *et al*., 2008). Hemimetabolous insect juveniles are capable of leg regeneration much like vertebrates, but studies have suggested that only some, and not all, genes involved in normal embryonic leg development might be involved in re-patterning nymphal legs (Nakamura *et al*., 2008). In fact, although the cricket *Gryllus bimaculatus* may have a morphologically similar leg development as a vertebrate animal, the signaling molecules and genes involved during development and regeneration are much more similar to another insect like the fruit fly than to other vertebrates (Nakamura *et al*., 2008). Therefore, despite appendage regeneration in insects being divided into

the same basic phases as regeneration in vertebrates consisting of wound healing, blastema formation, and patterning followed by growth, there are key differences in regeneration abilities and mechanisms between vertebrates and invertebrates (Truby, 1983, Nakamura *et al*., 2008).

The insect model system was used extensively for the study of regeneration mechanisms until the 1980s, yet very few groups currently work on the system due to the scarcity of molecular data and the lack of functional analysis tools. Despite these setbacks, however, improved sequencing techniques and the development of new molecular techniques, such as RNA interference (RNAi), have provided scientists with increasing information on insect re-patterning and cell organization (Nakamura *et al*., 2008).

Based on molecular studies done on the flour beetle, *Tribolium Castaneum*, two alternative hypotheses have been proposed for the origin of regeneration: an independent origin of limb regeneration mechanisms across metazoans and a common origin of blastema formation mechanism for all Metazoans. Several key pathways used in vertebrate blastema development also appear to be used for blastema growth in *Tribolium*. In this study, we focus on Hh signaling to determine its role during limb regeneration.

Hedgehog signaling in insects

In normal development, the *Drosophila* leg is divided into three distinct domains: posterior, dorsal/anterior, and ventral/anterior (Nakamura *et al*., 2008). These three compartments are characterized by distinct cell populations founded in early development. This compartmentalization of the leg into three circumferential cell populations has led to the development of the molecular boundary model. The molecular boundary model predicts that boundaries between cell populations define the presumptive distal tip of the leg and are the

source of diffusible morphogens that induce outgrowth and specify cell fate along the P/D axis (Campbell & Tomlinson, 1995). In *Drosophila*, it has become known that posterior cells involved with organizing growth and cell patterning in these domains function by secreting Hedgehog (Hh) protein, a known morphogen. Hh affects neighboring anterior cells by inducing them to secrete Decapentaplegic (Dpp) or Wingless (Wg) protein at the dorsal A/P and ventral A/P boundaries respectively (Campbell *et al*., 1993; Basler & Struhl, 1994; Diaz-Benjumea *et al*., 1994). A similar pattern of localization of proteins, such as Hh, Dpp, and Wg, has been found in *Gryllus* limb bud development. The gene expression pattern in both hemimetabolous and holometabolous insects provides evidence for the importance of these proteins in the formation of the P/D axis in insect legs (Nakamura *et al*., 2008).

The appendages of *Drosophila* starkly contrast the appendages of *Gryllus*, the hemimetabolous insect discussed earlier, in terms of their appendage development. *Drosophila* appendages arise from asymmetrical imaginal discs, which are compilations of cells comprised of a single epithelial sheet (Marsh & Theisen, 1999). Imaginal discs are divided into four domains identified by cell populations expressing *cubitus interuptus (ci*), *dpp*, *hh*/*engrailed (en*), and *wg*. In *Drosophila*, asymmetry in the dorsal/ventral (D/V) axis is generated by the expression of *dpp* dorsally and *wg* ventrally (Raftery *et al*., 1991; Couso *et al*., 1993). Many experiments have demonstrated that localized expression of just these two morphogens, Dpp and Wg, is sufficient to produce symmetrical limb outgrowth and normal pattern formation in *Drosophila* (Marsh & Theisen, 1999). However, loss of one or the other causes defects in patterning and improper growth. For example, loss of *wg* causes the loss of ventral structures and the duplication of dorsal structures while loss of *dpp* causes the loss of dorsal elements and the duplication of ventral elements (Baker, 1988; Struhl & Basler, 1993; Held *et al*., 1994).

Continuous input of these morphogens is essential throughout development and during the initiation of new patterning events in regeneration.

Unlike the basic network maintaining D/V axis asymmetry, A/P axis asymmetry is maintained by a network of selector genes controlling cell lineage (Blair, 1995). The posterior compartment is the controlling center, responsible for modulating expression of anterior genes and secreted morphogens. The homeotic selector gene *en* inhibits expression of *dpp*, *wg*, *ci*, and *ptc* (*patched*) while promoting expression of Hh (Sanicola *et al*., 1995; Zecca *et al*., 1995). The two key morphogens, *dpp* and *wg*, are located at the boundary between the anterior and posterior compartments and are regulated by Hh activity (Marsh & Theisen, 1999). Hh is expressed only in the posterior compartment but it maintains the ability to diffuse a short distance into the anterior compartment to interact with two other proteins, Smoothened (Smo) and Patched (Ptc). Smo is a seven-pass transmembrane protein which transduces Hh signaling to promote dpp (Alcedo *et al*., 1996). However, in the presence of Ptc, the Hh receptor, Smo activity is inhibited, and *dpp* and *wg* expression is decreased in the anterior compartment (Nakano *et al*., 1989; Chen & Struhl, 1996; Marsh & Theisen, 1999). In *Drosophila*, Hh regulates morphogen expression by binding to the Ptc receptor, causing Ptc inhibition and Smo activation (Fig. 1) (Chen & Struhl, 1996). This binding of Hh to Ptc is also responsible for creating the narrow region of *dpp* and *wg* expression seen between the anterior and posterior compartments. The anterior compartment in *Drosophila* is characterized by the expression of Ci, a zinc finger transcription factor (Dominguez *et al*., 1996). Ci can be produced as two different forms inside the cell: Ci75 and Ci155. At the A/P boundary, Hh signaling causes Ci to be converted into its activator form of Ci155, promoting the expression of *ddp*, *wg*, and *ptc* (Aza-Blanc *et al*., 1997; Ohlmeyer & Kalderon, 1998). Conversely, in the absence of Hh or in distant cells unable to

receive the Hh signal, Ci is converted to the repressor form of Ci75, which eventually inhibits *hh*, *dpp*, and *wg* expression (Dominguez *et al*., 1996; Aza-Blanc *et al*., 1997; Ohlmeyer & Kalderon, 1998). In *Drosophila*, loss of Hh function results in the loss of distal leg elements and truncated legs due to the altered expression of Wg and Dpp (Marsh & Theisen, 1999). Therefore, the ability to maintain the A/P axis during development appears to be highly contingent upon Hh signaling.

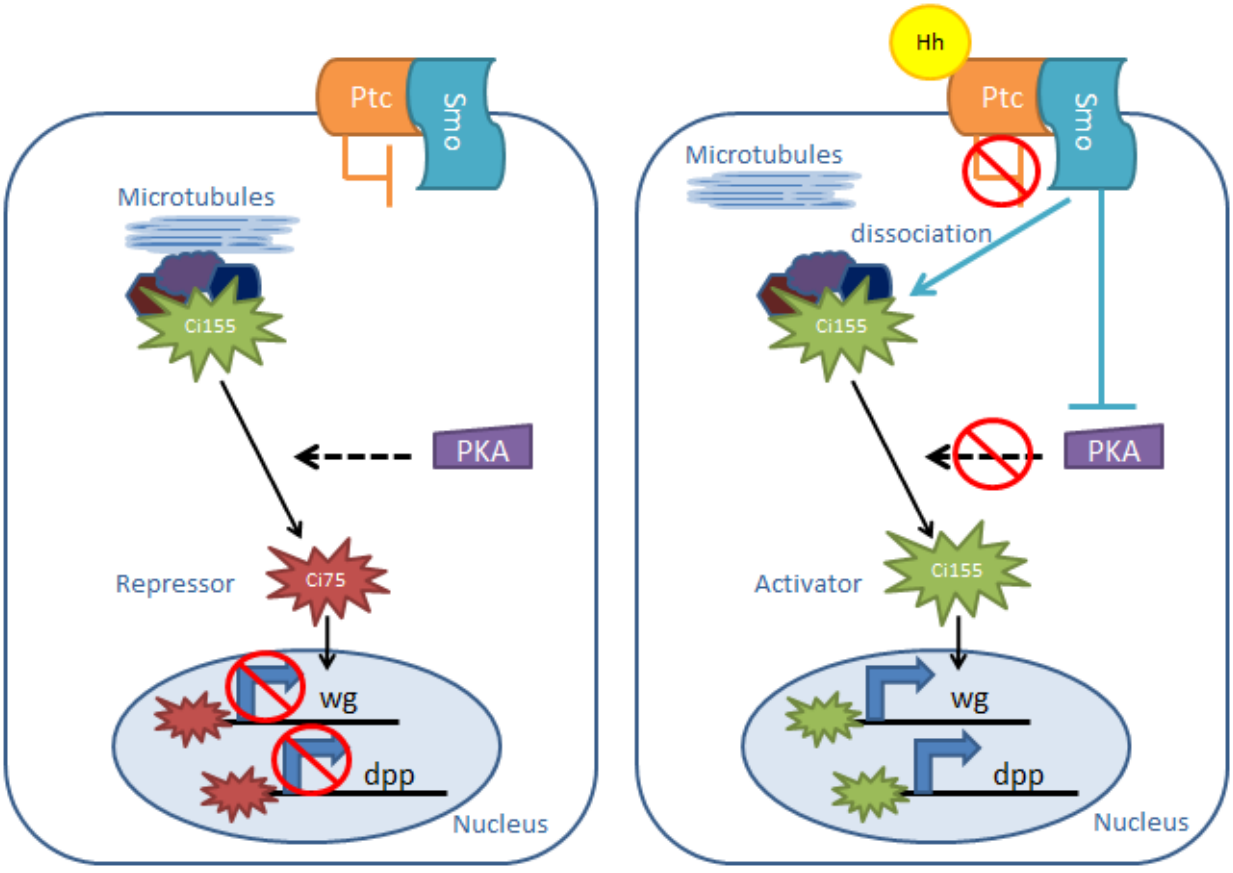

Figure 1: Hedgehog signaling in *Drosophila.* In the absence of Hh signaling, Ptc inhibits Smo activity (left cell). This allows Ci155 to be phosphorylated by PKA and cleaved to form Ci75, which is a transcriptional repressor, inhibiting *wg* and *dpp* transcription. In the presence of Hh signaling, Ptc is inactivated and Smo is activated (right cell). Smo inhibits PKA and promotes the release of Ci155, a transcriptional activator of *wg* and *dpp* expression.

Hedgehog pathway during regeneration

Hh, Dpp, and Wg expression patterns have been examined in insect regenerating limbs. Using RNA interference (RNAi) as the method of gene silencing, each of the three genes was individually silenced in *Gryllus* nymphs after leg amputation. In nymphs lacking Hh expression, regeneration is critically affected while silencing of Dpp and Wg has no phenotypic effects in either normal development or during nymphal leg regeneration (Nakamura *et al*., 2008). In contrast, the knockdown of Hh expression causes abnormal regeneration, indicated by the formation of a supernumerary axis (Nakamura *et al*., 2008). Therefore, Hh plays a critical role in insect limb patterning and proper re-patterning of regenerating limbs.

In this study, we examined the role of Hh during limb regeneration in a holometabolous insect whose legs and antennae undergo two major morphogenetic transformations during its life cycle: embryonic to larval and larval to adult via metamorphosis (Shah *et al*., 2011). The latter phase of morphogenesis transforms a morphologically simple larval appendage with few segments into a more complex adult appendage with many additional segments. Due to the unique role that the blastema plays across fundamentally most regenerating species, investigating the importance of signaling molecules and morphogens could provide key insights on the mechanisms governing the initiation of regeneration in other species. The goal of our study is to investigate the role that Hh plays during regeneration to determine what role it plays during limb regeneration in *Tribolium*.

Tribolium Castaneum life cycle

In this study, the red flour beetle, *Tribolium Castaneum*, was used to investigate the role of Hh signaling during limb regeneration. Holometabolous insects, like *Tribolium Castaneum*, are

characterized by three distinct life-cycle stages: larval, pupal and adult (Park, 1934). Each life history state is associated with a distinct morphology thought to permit the insect to inhabit different habitats and perform altered functions, such as growth in the larval stage, tissue reorganization in the pupal stage and reproduction in the adult (Suzuki *et al*., 2009). The larval stage is characterized by a series of molts that allow exponential insect growth over a period of approximately seven to eight instars (intervals between molts) (Park, 1934). Growth in the larval stage is accompanied by relatively few changes in morphology between instars (Suzuki *et al*., 2009). However, a dramatic change in morphology occurs at the end of the larval stage when metamorphosis is initiated. Metamorphosis is a marked change between larval and adult forms that occurs in two basic steps: from larval to pupal and from pupal to adult (Sehnal, 1996). The resulting pupa is largely immobile, does not feed and possesses inoperative wing pads demonstrating very little resemblance to the larva. During a relatively short period of time, dramatic tissue reorganization occurs in the pupa leading to the formation of adult structures and the ultimate transition into the adult stage (Suzuki *et al*., 2009). The short life cycle, sequenced genome, amenability to functional analyses using RNA interference (RNAi) and limb regeneration ability of *Tribolium* make it an excellent model system for the study of signaling factors during appendage regeneration.

Regeneration in Tribolium Castaneum

In most insects, appendage regeneration abilities are limited to the juvenile stage. This limitation is due to the requirement of molting for proper appendage growth (Shah *et al*., 2011). Certain insect models, like *Drosophila*, introduce obstacles in regeneration studies due to their presumptive leg development from imaginal discs inside the larval body, making regeneration examinations and analyses difficult to perform (Nakamura *et al*., 2008). Regeneration can be

more easily examined in insects like *Tribolium* that develop limbs externally from limb buds (Beermann *et al*., 2001). Therefore, limb regeneration in *Tribolium* provides an opportunity to investigate the functions of genes in an easily accessible system. When *Tribolium* larval legs are ablated at the onset of the sixth instar, larvae regenerate much of the leg segments within two molts; after three molts, the original leg morphology is restored making regeneration analyses quick and efficient (Shah *et al*., 2011). Additionally, molecular and genetic studies with *Tribolium* have improved over the last decade due to the complete sequencing of its genome (*Tribolium* Genome Sequencing Consortium, 2008). Interestingly, *Tribolium* contain a more ancestral representation of regulatory genes compared to *Drosophila*, making gene regulatory interactions in *Tribolium* more representative of those found in vertebrates than in other insects (Mitten *et al*., 2012). Given the availability of the sequenced genome and the ease of identifying orthologs of interest, working with *Tribolium* allows us to easily conduct functional analyses through techniques, such as RNAi.

RNA interference

One of the most important relationships in biology is that of structure and function. In genetics, determination of gene function is accomplished by silencing or knocking down the gene and noting phenotypic or physiological differences (Fortunato & Fraser, 2005). Most recently, RNAi has been incorporated into genetic studies in order to determine the impact of gene silencing on function. RNAi was first characterized in *C. elegans* by introduction of double stranded RNA (dsRNA) (Fire *et al*., 1998). It was also determined that only sequences coding for exons impacted gene silencing, which verified the effect of dsRNA on only processed mRNA. Not all genes are susceptible to these effects however, for example neuronal genes prove to be resistant to RNAi (Simmer, *et al.*, 2002). Further studies on the mechanisms of

RNAi have shown that dsRNA can be transported from any part of the organism via transmembrane proteins in cells (Winston *et al*., 2002; Feinberg & Hunter, 2003; Tomoyasu & Denell, 2004). This insight allows RNAi to be administered in a variety of ways including through diffusion by soaking, feeding and injections (Tomoyasu & Denell, 2004; Fortunato & Fraser, 2005). Our study incorporates the final method of injecting dsRNA into *Tribolium* in order to determine the effects of specific gene silencing on function. Using RNAi as a targeted gene knockdown technique allows for the ease of testing gene functionality during *Tribolium* larval leg regeneration.

Hedgehog signaling in Tribolium limb regeneration

In this study, the roles of the Hh signaling pathway was examined during *Tribolium* limb regeneration. Hh is an important growth regulator found most likely in every animal species, including humans. The *hh* gene was originally isolated from *Drosophila,* but soon homologs were found in many other species including vertebrates (Stark *et al*., 1998). The Hh pathway has been closely associated with cell cycle regulation, expansion of hematopoietic cells, D/V patterning of the neural tube, spermatogenesis, and as a negative regulator chondrocyte differentiation (Stark *et al*., 1998; Trowbridge *et al*., 2006).

While the mechanisms underlying regeneration are not well understood, there are many elements of regeneration that appear to be conserved between different species. As mentioned before, blastema formation is crucial to the regeneration process because without a blastema regeneration cannot be completed (Singh *et al*., 2012). Understanding the mechanisms behind blastema formation will allow us to apply this knowledge to other organisms including humans.

How does the regenerating tissue know what to become? What determines the size and proper growth of this tissue or appendage?

In the present study, we analyzed the function of the Hh signaling pathway during larval limb regeneration in *Tribolium* to determine whether Hh signaling plays a conserved role in the regeneration process. Although expression of Hh is known to be crucial for regeneration in amphibians, the exact role and mechanism of this signaling factor is unclear, especially within insect models. Through leg ablations and RNAi knockdown of Hh, we analyzed the role of Hh signaling during leg regeneration in *Tribolium*.

MATERIALS AND METHODS

Beetle husbandry

Wildtype *Tribolium Castaneum* strain GA1 was obtained from Dr. Richard Beeman (USDA ARS Biological Research Unit, Grain Marketing & Production Research Center, Manhattan, Kansas). All beetles were raised on organic whole wheat flour containing 5% nutritional yeast and kept in an incubator at a constant temperature of 29ºC and 50% relative humidity.

RNA isolation

In order to amplify and clone cDNA from *Tribolium* larvae for eventual dsRNA synthesis and injection, RNA was isolated from *Tribolium* at random larval stages. Larval tissue was dissected in 1X-phosphate-buffered saline (PBS; 0.02 M phosphate, 0.15 M NaCl, 0.0038 M NaH_2PO_4, 0.0162 M Na_2HPO_4; pH 7.4) to remove the fat body and gut. The remaining tissue was homogenized in TRIzol (Invitrogen) and treated with chloroform in order to isolate RNA. The RNA was sequentially treated with RQ1 RNase-Free DNase (Promega) according to the manufacturer's instructions.

cDNA synthesis and polymerase chain reaction

cDNA was synthesized from 1 μg of RNA using the First Strand cDNA Synthesis Kit (Fermentas) according to the manufacturer's instructions. The sequence for *hh* (GenBank accession number NM_001114365) was obtained from GenBank and Beetlebase. Primers for this gene were designed (Table 1) and used to amplify cDNA through a polymerase chain reaction (PCR). PCR products were then analyzed by agarose gel electrophoresis. cDNA was

extracted from the gel using the MinElute Gel Extraction Kit (Qiagen) according to the manufacturer's instructions.

Table 1. Primer sequences used in this study

Gene	**Direction**	**Sequence (5'->3')**	**Product Size (bp)**
hh	Forward	CCTCTCCTCGCTCCAAATC	605
	Reverse	CAAAGGTCTATCCGCACTACC	
hh (knockdown verification)	Forward	ACTGCTCGGTCAAATCAGAA	713
	Reverse	TAAGGAACACTCAAAGGTCTATCC	

Cloning and dsRNA synthesis

Isolated PCR product was cloned into the pCR-4 TOPO-TA vector (Invitrogen). Plasmids from the cloned vector were purified using the QIAprep Spin Miniprep Kit (Qiagen) according to the manufacturer's instructions. After the verification of the inserts through sequencing, the plasmids were digested using Spe1 and Not1 restriction enzymes (NE Biolabs, Ipswich, MA).

Single-stranded RNA (ssRNA) was synthesized using the T3 and T7 MEGAscript Kits (Ambion) according to the manufacturer's instructions. Equal amounts of complimentary ssRNA were then used to create a 2 μg/μl solution of dsRNA in diethyl pyrocarbonate-treated water. dsRNA was annealed as described previously by Hughes and Kaufman (2000). The annealed product was analyzed by agarose gel electrophoresis and compared to ssRNA to confirm proper annealing.

dsRNA injections

dsRNA was either injected into day zero fifth or sixth instar larvae prior to limb ablation or into day zero seventh instar larvae one or two molts after limb ablation, depending on the treatment group. Approximately 0.5 μg (0.25 μl) of *hh* or *wnt-1* dsRNA was injected into the dorsal side of all larvae using a pulled 10-μl glass capillary needle and a 50-ml syringe. For control animals, the same amount of bacterial *ampicillin resistance* (*amp*^{r}) dsRNA (plasmid obtained from Dr. Takashi Koyoma, the Gulbenkian Institute of Science, Portugal) was injected. Following injections, larvae were maintained in individual plastic cups with whole wheat flour at normal conditions. Phenotypes were observed in four day increments after injection and daily after the pre-pupal stage.

Leg ablations

Larval mid- and hind legs were ablated two days following injections to ensure that the RNAi-mediated knockdown was in full effect. Larvae anesthetized on ice were placed ventral side up and mid- and hind-legs were cut close to the base of the femur. All cuts were made with fine microscissors under a dissecting microscope. The forelegs and the contralateral mid- and hind-legs served as internal controls for regeneration analysis. Following leg ablations, larvae were kept in normal conditions, and observations on regenerating legs were recorded after each molt in the larval stage as well as after metamorphosis into the pupa. Animals were stored in a solution containing 15% glycerol and 70% ethanol at -20ºC until imaged. Larval legs of both *hh* and *amp*^{r} control animals were then dissected and mounted in an 80% glycerol solution. Because larval leg tissues produce their own autofluorescence, mounts were examined using a Nikon Eclipse 80i fluorescence microscope. Images were taken using a QImaging camera

(Diagnostic Instruments) and NIS Elements Imaging Software. All images were compiled using ImageJ.

Two other treatment groups were created in which the order of injections and ablations were reversed. The first treatment group consisted of non-injected day 0 sixth instar larvae. Larval mid- and hind legs were then ablated two days later following the same procedure as described above. Following leg ablations, larvae were kept in normal conditions and monitored continuously until the first larval molt. On day 0, succeeding the first larval molt after leg ablations, larvae were injected with dsRNA. The second treatment group consisted of non-injected day 0 fifth instar larvae. Larval mid- and hind legs were ablated from these animals two days later and larvae were kept in normal conditions until the second larval molt. Immediately after the second larval molt after leg ablations, larvae were injected with dsRNA. Following all injections, larvae were kept in normal conditions, and observations on regenerating legs were recorded after each molt in the larval stage as well as after metamorphosis into the pupal stage. All animals were stored in a solution containing 15% glycerol and 70% ethanol at -20ºC until imaged. Larval legs of both *hh* and *amp*^{r} dsRNA-injected animals were then dissected one molt after injections and mounted in an 80% glycerol solution. Day 0 pupae were also stored and imaged using fluorescence microscopy.

Antenna ablations

Similar procedures were used for antenna regeneration studies as those used for the leg regeneration studies. Larval antennae from the left side of the head were ablated using a razor blade. The contralateral antenna served as the internal control. Following antenna ablations, larvae were kept in normal conditions, and observations of regenerating antenna were recorded

after each molt in the larval stage as well as after metamorphosis into the pupal stage. Animals were stored and larval antennas of both *hh* and *amp*^{r} control animals were dissected and mounted in the same manner as described above. Antenna mounts were examined using a Nikon 50i Trinocular Microscope. Images were taken using an 18.2 Color Mosaic camera (Diagnostic Instruments) and SPOT Advanced software. All images were compiled using ImageJ.

BrdU staining of blastema cells

In order to study cellular proliferation during regeneration, BrdU staining was performed on blastema structures. Wildtype, *hh* dsRNA-injected and *amp*^{r} dsRNA-injected sixth instar larvae were all stained with BrdU one molt after leg ablation. The staining procedure began with an initial dissection of individual larval segments containing blastema structures. Segments were isolated in PBS using a sharp razor blade, and internal tissues were removed as best as possible, without disrupting the outside cuticle. The remaining epidermis and blastema structures were incubated for three hours in 20 μg/μl BrdU in PBS. The solution was then removed, and the tissues were fixed in 3.7% formaldehyde in PBS overnight at 4ºC. The tissues were then rinsed twice with a solution of PBS with 1% Triton-X 100 (PBS-TX). The tissues were then submerged in 2 N HCl in PBS-TX for one hour at 37ºC. Tissues were rinsed again and then blocked with 5% NGS in PBS-TX for 30 min at room temperature. Subsequently, the solution was removed, and tissues were incubated in 1:200 anti-BrdU antibody in PBS-TX overnight at 4ºC. The tissue was subsequently incubated in a solution of 1:1000 Alexa Fluor 488 goat anti-mouse antibody in PBS-TX overnight at 4ºC. Finally, tissues were rinsed a few times with PBS-TX and mounted with Vectashield for imaging. Mounts were examined using a Nikon Eclipse 80i fluorescence microscope, and images were taken using a QImaging camera (Diagnostic Instruments) and NIS Elements Imaging Software. All images were compiled using ImageJ.

Semi-quantitative RT-PCR and knockdown verification

Knockdown of *hh* mRNA expression in *hh* dsRNA-injected animals was verified through semi-quantitative reverse transcription-PCR (RT-PCR). Day zero seventh instar larvae were injected with *hh* dsRNA and two pre-pupa were used for RNA isolation. *amp*^{r} dsRNA-injected pre-pupa were used as controls. After RNA isolation, 1 μg of RNA from each treatment group was converted into cDNA and semi-quantitative RT-PCR was done on these samples. *Ribosomal protein 49* (*rp49*) (Konopova & Jindra, 2007) was used as a control to ensure that equal amounts of cDNA were used for each reaction. Thirty-four cycles were used for *hh* and *rp49*. Two replicate RT-PCRs were performed to ensure repeatability of the knockdown expression.

RESULTS

Hh participates in limb patterning and compound eye development

To characterize how Hh silencing affects normal development in *Tribolium*, *hh* dsRNA was injected into day zero sixth instar larvae, and treated animals were observed during all subsequent life stages. Of the larvae that survived past the first larval molt following injections (n=18), 30% (n=5) survived to the pupal stage, with the rest dying primarily during the larval molts and pre-pupal stage. The *hh* dsRNA-injected animals that survived to pupate died upon pupation (Fig. 2I). This finding suggests that Hh is required for proper development into the pupal and the adult stages.

Pupal limb morphology also became affected as a result of Hh silencing. The wings became severely reduced to small outgrowths (Fig. 2 I & J). Pupal terminal appendages also became rounded and diminished in size (Fig. 2M). Pupal antennae were considerably reduced in size compared to the control *amp*^{r} dsRNA-injected animals (compare Fig. 2 F and N). Pupal legs were severely reduced in width and some exhibited branching patterns that are not seen in normal pupal legs (Fig. 2 O-R). The affected leg segments lacked the normal tarsal and tibial morphology, and instead were threadlike and often contorted (Fig. 2 Q-R). The femur also appeared mildly affected; its morphology was more bulbous than the normal femur (Fig. 2 Q-R).

Eye development during the pupal stage was also drastically impacted by the silencing of Hh expression. Compound eyes normally begin to develop in day zero pupae. However, *hh* knockdown animals lacked any signs of compound eye development during this stage (Fig. 2L). Eyes retained in these animals resemble larval-like eye morphology, appearing as two dark eye spots, instead of a band of compound eyes, as was seen in the *amp*^{r} dsRNA-injected day zero

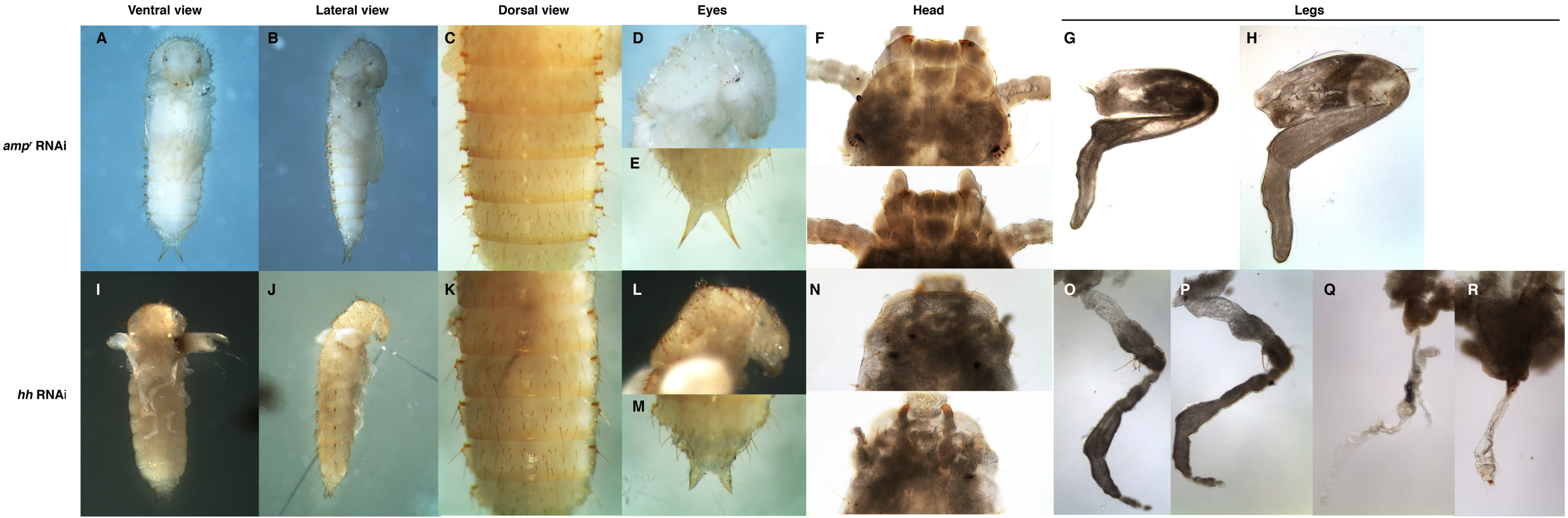

Figure 2: Effects of *hh* knockdown on the pupal phenotype of *Tribolium*. (A-H) Day zero control pupa injected with 2 µg/µL *amp*^{r} dsRNA at day zero sixth instar. (I-R) Day zero pupa injected with 2 µg/µL *hh* dsRNA at day zero sixth instar. Images shown are the whole body (A,B,I & J), gin traps (C & K), eyes (D & L), tail (E & M), dorsal head above ventral head (F & N), and mid- and hind-legs shown on the left and right, respectively (G,H,O,P,Q & R). (Q & R) Strongly affected day two Hh knockdown pupal legs

pupae (Fig. 2D). The gin traps in *hh* knockdown animals were also affected (compare Fig. 2 C to K). These lateral protruding structures on *Tribolium* pupae were less pronounced and in particular, the posterior portion of each gin trap was noticeably reduced (Fig. 2K). Together these results imply that Hh is required for proper adult limb patterning and compound eye development in *Tribolium*.

Knockdown of Hh expression prevents leg regeneration

To examine the role of Hh during *Tribolium* leg regeneration, *hh* dsRNA was injected into day zero sixth instar larvae. Two days later, the mid- and hind- legs on one side of the animal were ablated. Animals were observed every four days following leg ablations, and daily after the pre-pupal stage. In *amp*^{r} dsRNA-injected control animals, wound healing occurred and blastema-like structures formed after the first larval molt (Fig. 3A). After the second larval molt, segments were re-formed and the leg began to take on the general leg morphology (Fig. 3B). In the pupae, these legs were fully regenerated as long as the larva molted once before pupation.

In Hh knockdown animals, all larvae showed complete wound healing of their ablated legs similar to *amp*^{r} dsRNA-injected control animals after one molt (Fig. 3C). Along with wound healing, most of the *hh* dsRNA-injected larvae formed rounded blastema-like structures at ablation sites. After the second molt, none of the *hh* knockdown larvae showed any signs of leg regeneration. The rounded structures seen in the previous molt was the only apparent outgrowth (Fig. 3D), indicating that little cell proliferation had taken place at the ablated site. Similarly, larvae that developed into pupae after two molts (n=12) did not show any signs of regenerated pupal legs (Fig. 4B). The rounded mass of cells apparent during the larval stage disappeared in the pupal stage and instead, sites of prior leg ablation were completely devoid of

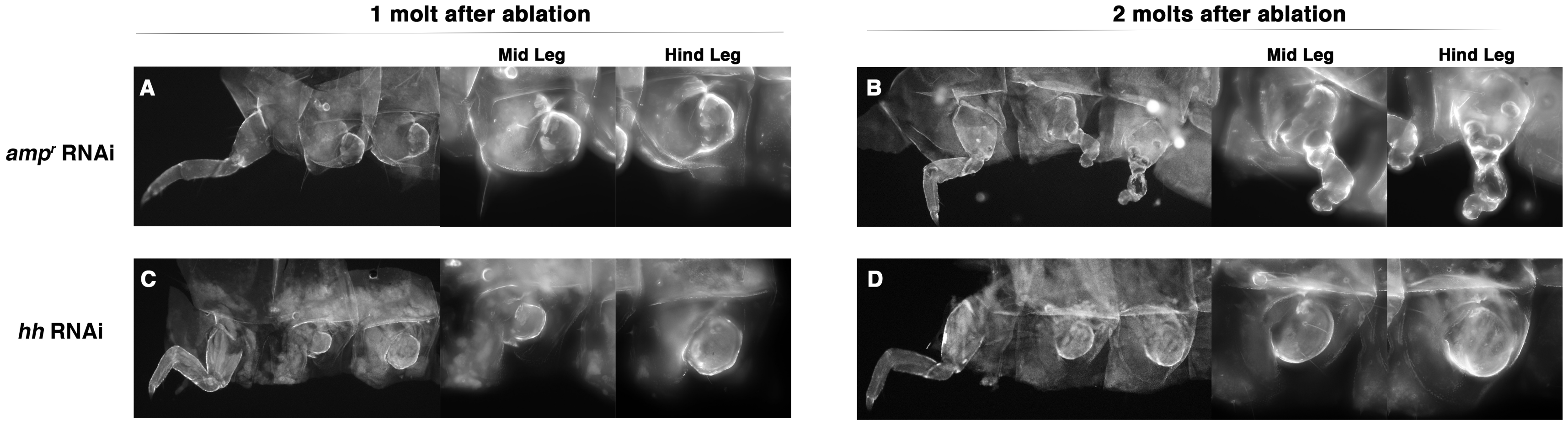

Figure 3: Effects of *hh* knockdown on *Tribolium* larval leg regeneration. All animals were injected with 2 µg/µL *amp*^{r} or *hh* dsRNA, and mid- and hind-legs were cut two days after. (A & C) Regenerating larval legs one molt after ablation. (B & D) Regenerating larval legs two molts after ablation. Middle panel shows the mid-leg and right panel shows the hind-leg.

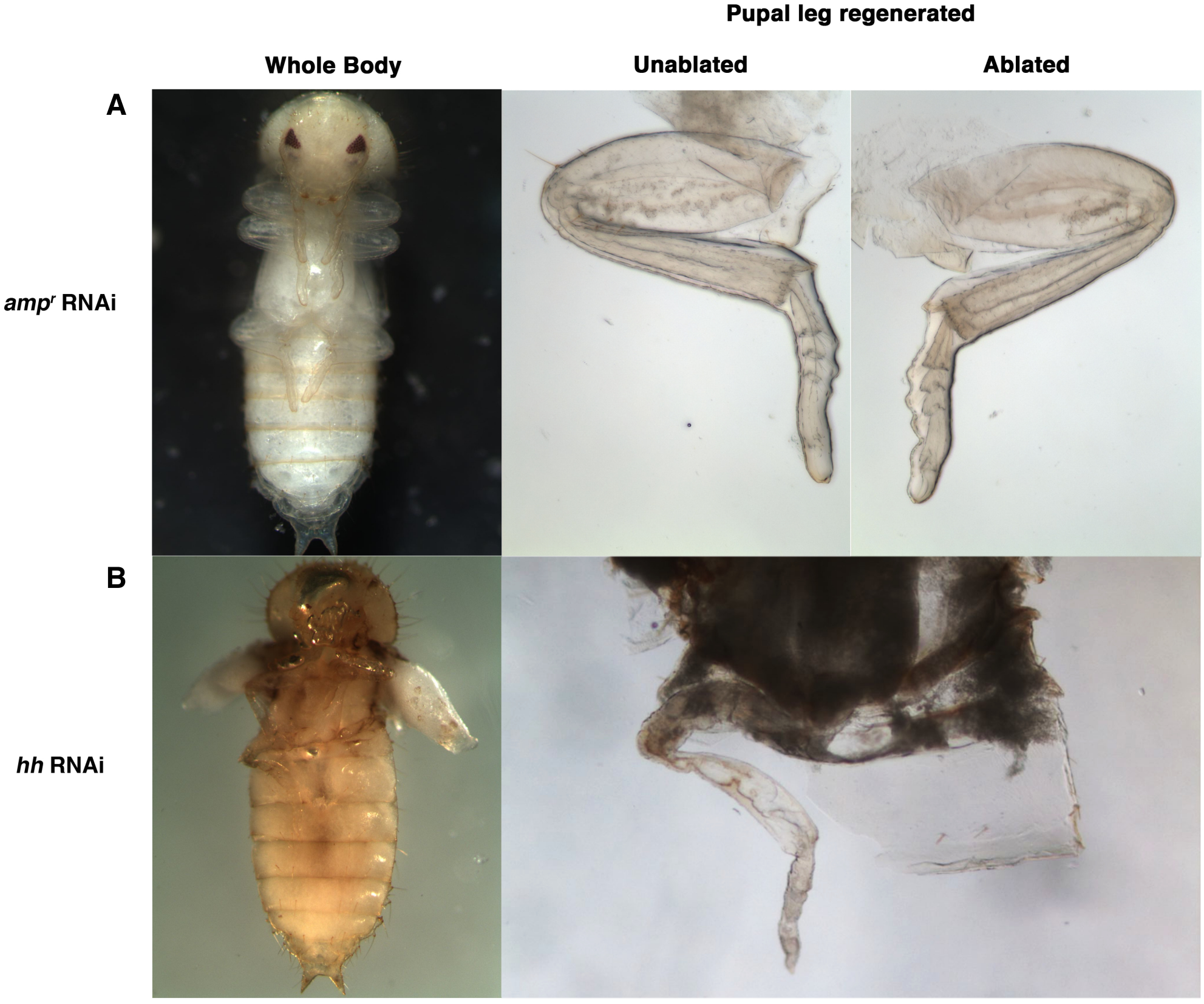

Figure 4: Effects of *hh* knockdown on pupal leg regeneration in *Tribolium*. (A) Control pupa injected with 2 µg/µL *amp*^{r} dsRNA at day zero sixth instar and mid- and hind-leg ablations performed two days later. Images from Shah et al. 2011. (B) Strongly affected pupa injected with 2 µg/µL *hh* dsRNA at day zero sixth instar. Mid- and hind-legs were ablated two days following injections. Whole body (left panel) and hind segment (right panel) are shown.

any limb structures as indicated by the flattened and smooth cuticle (Fig. 4B). Together these results indicate that while Hh is not necessary for wound healing and initial blastema formation, it is required for growth of the blastema cells and full regeneration in *Tribolium*.

Hh is essential throughout the entire regeneration process

In order to further investigate the role of Hh during the re-patterning phase that occurs after the blastema has formed, we delayed the time of injection until one or two molts after mid- and hind-leg ablations. In one group of animals, legs were ablated on day two sixth instar larvae, and animals were injected with dsRNA after one molt, when the wound had healed and the blastema had formed. A second group was set up in which sixth instar larvae ablated on day two and injected with dsRNA after two molts, when the leg segments were re-established but not yet perfect in morphology. In the *amp*^{r} dsRNA-injected control group, ablated legs continued to regenerate normally for both treatment groups (Fig. 5 A &B).

In *hh* dsRNA-injected animals, when the order of injection was reversed, larval legs still failed to regenerate (Fig. 5 C & D). In the first treatment group, injected with *hh* dsRNA after one molt, regeneration terminated at the blastema stage (Fig. 5C). No further leg growth occurred after *hh* knockdown as can be seen by the rounded blastema-like structure still present after the second larval molt (Fig. 5C). Thus, allowing for the wound site to heal and for the blastema to form before knockdown of Hh expression is not sufficient to promote regeneration in *Tribolium* larval legs. In the second treatment group, with Hh knockdown after the second molt, the regenerated larval legs after one additional molt was smaller than those seen in similarly treated *amp*^{r} dsRNA-injected larvae (Fig. 5D). The legs had proper number of segments. However, the resulting leg segments had distorted segment morphology unlike the unablated

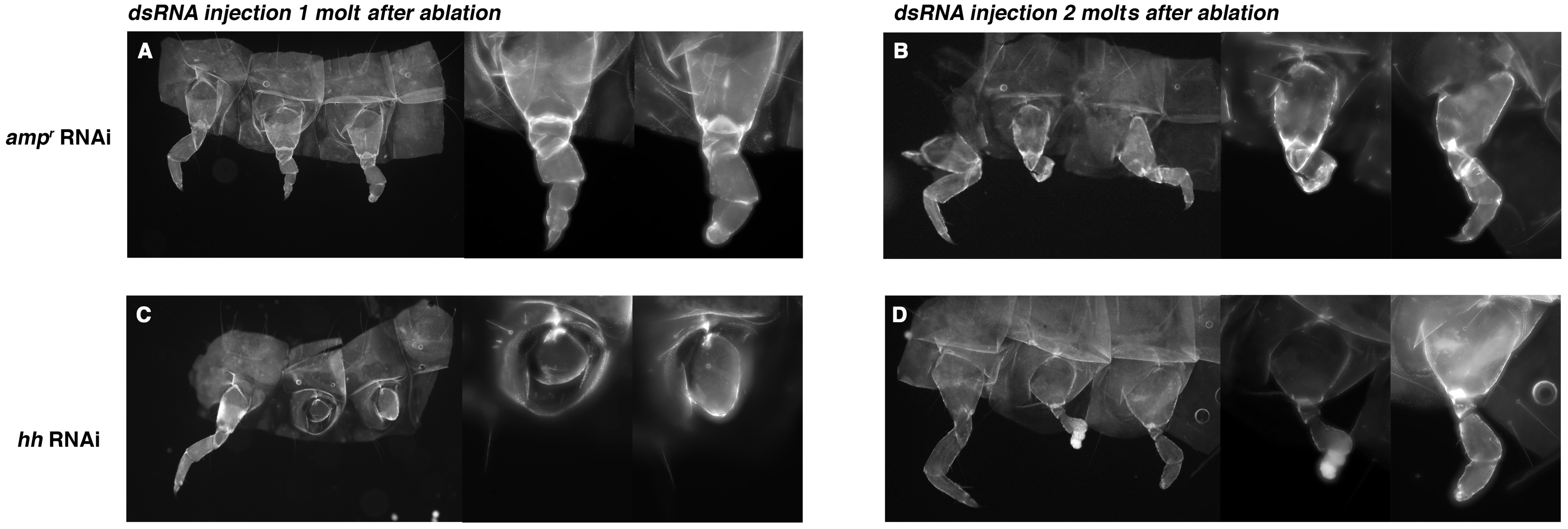

Figure 5: Effects of *hh* knockdown on *Tribolium* larval leg regeneration one and two molts after ablation. (A & C) Day 2 sixth instar larval legs were ablated and animals were injected with 2 µg/µL *amp*^{r} or *hh* dsRNA after the first larval molt. Regenerating larval legs two molts after ablation. (B & D) Day 2 sixth instar larval legs were ablated and animals were injected with 2 µg/µL *amp*^{r} or *hh* dsRNA after the second larval molt. Regenerating larval legs three molts after ablation. Middle panel shows the mid-leg and right panel shows the hind-leg.

foreleg and the regenerated *amp*^{r} control mid- and hind-legs (compare Fig. 5 B & D). These results indicate that Hh is involved in regulating cell proliferation and growth during leg regeneration in *Tribolium* larvae.

In order to elucidate the full effects of Hh knockdown on regeneration, pupal legs in both treatment groups were analyzed. The pupal leg of *amp*^{r} dsRNA-injected control animals in the first treatment group regenerated normally (data not shown). The pupal leg of *amp*^{r} dsRNA-injected control animals of the second treatment group, injected after two molts, regenerated normally after two molts (n=3) (Fig. 6A). In *hh dsRNA-injected* treated animals, the truncated larval legs present during the third molt completely disappeared in the pupa. Only a small remnant of a leg was apparent as a tiny bump of cuticle on the coxa (Fig. 6B). Thus, this finding suggests that Hh is required for proper larval leg regeneration and eventual transformation into adult legs after metamorphosis.

Knockdown of Hh expression prevents antenna regeneration

To examine the role of Hh during *Tribolium* antenna regeneration, *hh* dsRNA was injected into day zero sixth instar larvae, and the right antenna was ablated two days later. Animals were observed every four days following antenna ablations, and daily after the pre-pupal stage. Similar to leg regeneration, antennal regeneration required two molts to restore normal morphology and size of ablated antennae. In *amp*^{r} control animals, wound healing occurred and blastema-like structures formed after the first larval molt (Fig. 7A). Antenna regeneration then proceeded to reform all segments and restore the antenna to its proper size after the second larval molt (Fig. 7B). In Hh knockdown animals, larvae showed complete wound healing after one molt (Fig. 7C). A tiny blastema-like bump was observed at the ablation

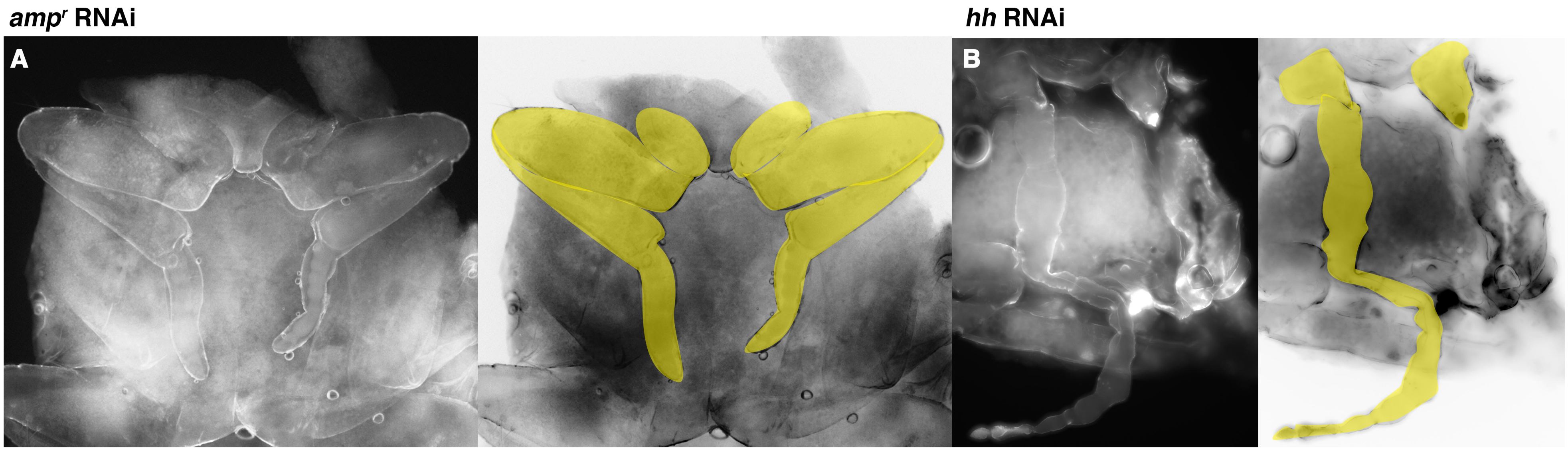

Figure 6: Effects of *hh* knockdown two molts after ablation on pupal leg regeneration in *Tribolium*. (A) Control pupa injected with 2 µg/µL *amp*^{r} dsRNA two molts after leg ablations. (B) Strongly affected pupa injected with 2 µg/µL *hh* dsRNA two molts after leg ablation. Mid segment (left panel) and color level inversion image of legs (right panel).

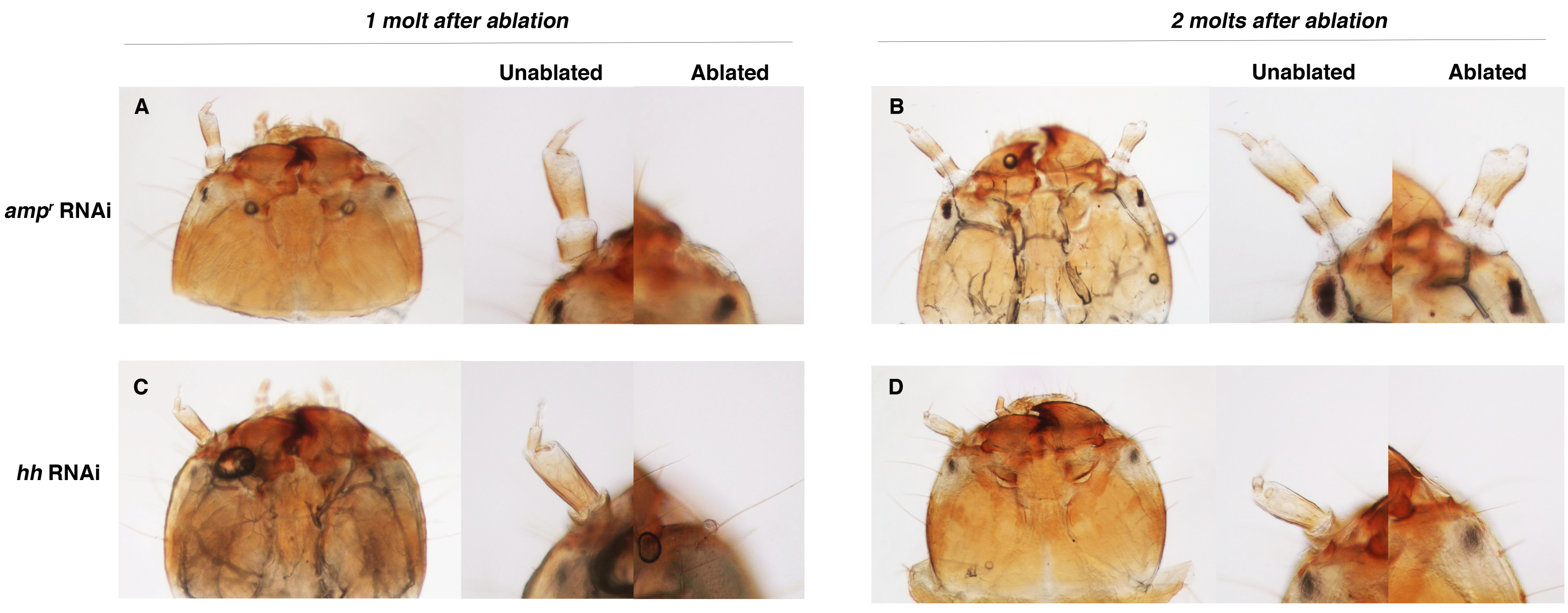

Figure 7: Effects of *hh* knockdown on larval antennal regeneration. Larvae were treated with 2 µg/µL *amp*^{r} (A & B) and *hh* (C & D) dsRNA. The left panels show the head of the treated larvae, the middle panels show the unablated antennae and the right panels show the ablated antennae.

site of larvae after one molt, indicating again that Hh is not required for regeneration initiation. After the second molt, however, none of the Hh knockdown larvae (n=16) showed any signs of antenna regeneration (Fig. 7D). The pupae also failed to regenerate their antennae (n=9; data not shown). Thus, Hh is required for antennal regeneration in *Tribolium*.

Hh plays an important role in blastema cell proliferation

To determine the period during which the most proliferation occurs, we examined blastemas in wild type *Tribolium* larvae daily after the first molt. The larvae at this stage typically molt after four to five days. Thus, day 0 to day 4 blastemas were stained with BrdU in order to identify proliferating cells. Day 4 blastemas contained a distinct population of large proliferating cells that were absent in the blastemas collected on other days (Fig. 8 A-E). On day 4, larger cells became apparent in the blastema, as opposed to the smaller cells that line the cuticle (Fig. 8E). We hypothesized that these cells might contribute to the regenerating limb. Therefore, we investigated in the effects of *amp*^{r}, *hh*, and *wnt-1* knockdown on blastema cell proliferation four days after the first molt.

Day two sixth instar larvae had their two hind-legs ablated and four days after their first molt, the blastemas were processed for BrdU staining. As a control, *amp*^{r} RNAi larvae were stained with BrdU and compared to *hh* RNAi larvae. In general, Hh knockdown diminished the number of proliferating large blastema cell compared to control (Fig. 8F), but cells were still proliferating. Next, blastema proliferation in *wnt-1* knockdown larvae was also analyzed. *Wnt-1* knockdown has been shown to prevent blastema formation (Shah *et al*., 2011). The *wnt-1* knockdown blastemas showed no large blastema cell proliferation although the smaller cells lining the cuticle incorporated BrdU staining (Fig. 8G). Thus, Hh appears to be required at least

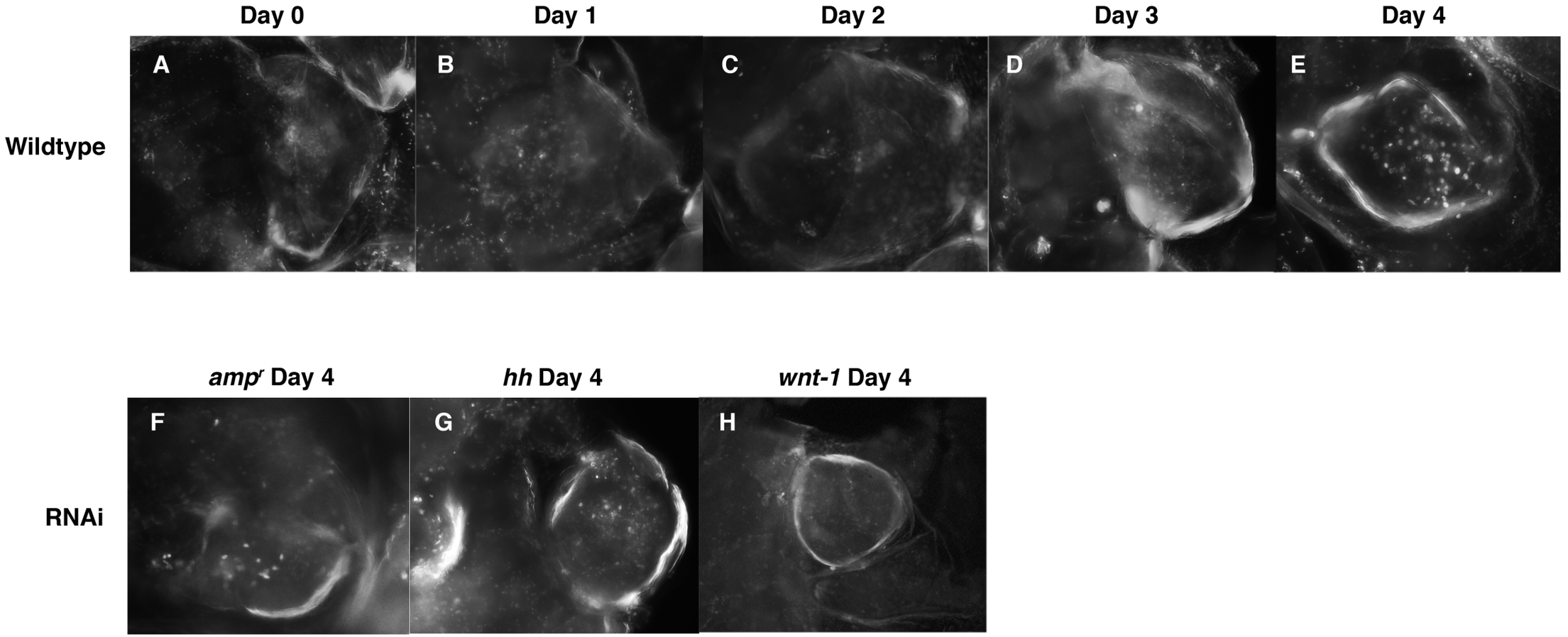

Figure 8: Blastema proliferation assays on wild type, *amp*^{r}, *hh* and *wnt-1* knockdown *Tribolium* larvae. (A-E) Day zero sixth instar larvae were collected and hind-legs were cut on day two. Animals were then processed 0, 1, 2, 3, and 4 days after the first larval molt. (F-G) Day zero sixth instar larvae were injected with 2 µg/µL *amp*^{r} (F), *hh* (E) and *wnt-1* (G) dsRNA, and hind-legs were cut on day two. Larvae were processed four days after the first larval molt. All blastemas were stained with BrdU in order to determine blastema-cell proliferation.

partially to maintain high levels of blastema-cell proliferation although Wnt-1 may have a greater role.

Confirmation of Hh *knockdown*

In order to verify that the dsRNA-injections resulted in the corresponding gene knockdown, a semi-quantitative RT-PCR was performed. Seventh instar larvae were injected on day zero with either *amp*^{r} or *hh* dsRNA. cDNA from all animals was collected during the pre-pupal stage and used for knockdown verification. The RT-PCR verified gene knockdown for both *amp*^{r} and *hh*, confirming that the phenotypes observed were due to knockdown of the corresponding gene (Fig. 9).

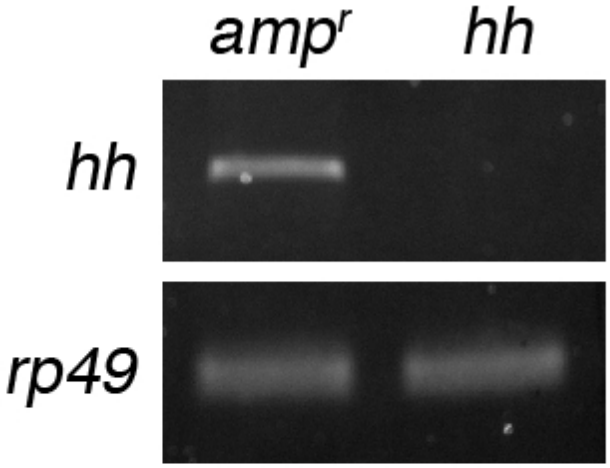

Figure 9: Knockdown verification of dsRNA injected larvae. Day zero seventh instar larvae were injected with 2 μg/μL *amp*^{r} and *hh* dsRNA and total RNA was isolated from pre-pupae. The cycle number for *hh* and *rp49* was 34 cycles.

DISCUSSION

In this study, the role of Hh was investigated during normal development and during leg and antenna regeneration using RNAi. Hh knockdown resulted in patterning defects in appendages and eyes during metamorphosis and subsequent death at the pupal stage. Hh was also found to be essential for both leg and antenna regeneration. Larvae were able to form blastema-like structures after ablations; however these structures were unable to grow and repattern into functional appendages even after multiple larval molts. Upon metamorphosis, these pupae still lacked limbs at the site of ablation. In addition, we found that administering RNAi after the onset of leg regeneration also prevented complete restoration of larval leg morphology, and upon metamorphosis, the regenerated leg structures were lost. Lastly, Hh knockdown resulted in reduction of cell proliferation in the blastema. Combined, these results indicate that Hh is required for adult limb patterning, limb regeneration, and blastema cell proliferation in *Tribolium*.

Hh is required for metamorphic remodeling of larval appendages

Knockdown of *hh* expression disrupted leg patterning during the pupal stage as well as compound eye development (Fig. 2). Our results indicate that Hh is important for normal pupal appendage development which is consistent with previous studies on the role of Hh in *Drosophila*. In *Tribolium*, knockdown of Hh causes pupal legs become threadlike and contorted (Fig. 2 O-R). In addition, ectopic branches were seen on some appendages, indicating that polarity might also be affected. These findings are consistent with limb patterning studies done in *Drosophila* which also show that *hh* is required for proper patterning and growth (Ingham, 1995; Ingham and Fietz, 1995). For example, in the *Drosophila* wing, Hh causes respecification of anterior wing compartments, causing some proximal anterior structures to disappear

completely (Ingham and Fietz, 1995). During the *Tribolium* pupal stage, the development of compound eyes is also inhibited in the absence of Hh expression (Fig. 2L). In *Drosophila*, Hh also plays a key role in the initiation of morphogenetic furrow, which is required for the differentiation of compound eye development (Borod and Heberlein, 1998; Pappu *et al*., 2003).

Hh regulates blastema growth and blastema cell proliferation

Our study shows that Hh plays an essential role in leg and antenna regeneration. In Hh knockdown animals, larvae showed complete wound healing and the formation of rounded blastema-like structures at the ablated sites. However, none of the Hh-knockdown larvae showed any signs of leg or antenna regeneration after the second molt, and cell proliferation was reduced in the blastema (Fig. 3 & Fig. 7). Pupae also lacked signs of leg or antenna regeneration (Fig. 4). Our results differ from reported phenotypes seen in crickets (Nakamura, 2008). In *hh* dsRNA-injected cricket nymphs, supernumerary legs form during regeneration indicating that Hh affects limb patterning by disrupting the positional information predicted by the molecular boundary model (Nakamura, 2008). Thus, while crickets can form blastemas and begin repatterning in the absence of Hh, *Tribolium* requires Hh for blastema growth and proliferation.

The role of Hh during *Tribolium* limb regeneration appears to be more similar to that during vertebrate limb regeneration. In amphibians, Shh regulates the cellular proliferation and migration of progenitors during limb regeneration (Signh *et al*., 2011). Shh and Ihh are also expressed early in the regenerating blastema, indicating that Hh signal governs the initial stages of regeneration (Singh *et al*., 2011; Stark *et al*., 1998; Endo *et al*., 1997; Imokawa and Yoshizato, 1997). Moreover, inhibition of the Hh signaling in amphibians results in stump formation and lack of regeneration, similar to our findings in *Tribolium* (Signh *et al*., 2011).

Furthermore, Hedgehog expression is also elevated in regenerating zebrafish fins (Avaron *et al*., 2006). This implies that the role of Hedgehog signaling during regeneration may be conserved in both vertebrates and invertebrates.

Regulation of blastema cell proliferation

The BrdU study shows that there appears to be special group of cells in the blastema that proliferate at the end of the intermolt period. These cells are visible by the larger nuclear staining of BrdU. We think that these are the cells that contribute to the regenerating appendage as knockdown of key regulators of blastema growth, Wnt-1 and Hh, inhibited or partially inhibited their proliferation. Compared to control amp^r RNAi larvae, *hh* RNAi larvae had reduced proliferation of these larger cells (Fig. 8F). In contrast, *wnt-1* knockdown larvae completely lacked proliferation of these cells (Fig. 8G). Given that the effects of knockdown on proliferation correlate with the degree of blastema growth, we think that the large cells are likely the major contributors that recreate the regenerating legs. We think that Hh is at least partially required to maintain high levels of blastema-cell proliferation whereas Wnt-1 is indispensable for blastema growth and proliferation.

The observation that these cells only proliferate at the end of the intermolt period suggests an intriguing hypothesis that the proliferation of these cells might be under the control of endocrine regulation. The molting hormones, ecdysteroids, are secreted prior to a molt and play major roles in cell proliferation (Gunamalai *et al*., 2004; Hopkins *et al*., 1999). Of particular interest is the fact that steroid hormones, such as ecdysone, play important roles during blastema cell proliferation in crustaceans (Das and Durica, 2013). In fiddler crabs, knockdown of ecdysteroid receptor inhibits blastema development and cell proliferation. Thus, signaling via the

ecdysteroid receptor pathway is necessary for blastemal cell proliferation and development in the regenerating limbs of crabs (Das and Durica, 2013). *Tribolium* blastema cell proliferation may also rely on ecdysteroids during regeneration. Future studies in *Tribolium* should address the interactions between Hh, Wnt and ecdysteroid signaling in limb regeneration.

The role of Hh and imaginal discs in larval to adult leg transformations

The phenotypes obtained by delaying the time of *hh* dsRNA-injection until one or two molts after leg ablations revealed unexpected insights on larval-to-adult leg transformation in *Tribolium*. In *hh* dsRNA-injected animals, larval legs failed to regenerate in both treatment groups (Fig. 5 C & D). Thus, allowing for the blastema to form and begin re-patterning before knockdown of Hh expression is not sufficient to promote regeneration in *Tribolium* larval legs. However, the results of the second treatment group are particularly noteworthy. In these animals, while the regenerated larval legs were apparent albeit somewhat distorted after the third larval molt post ablation, the pupae lacked legs. Only a small remnant of a leg was apparent as a tiny bump of cuticle on the coxa (Fig. 6B). These results suggest that in addition to its role in proper larval leg regeneration, Hh appears necessary for producing cells that eventually contribute to the adult legs during metamorphosis.

The disappearance of the partially regenerated larval legs implies the existence of imaginal cells in *Tribolium*. Previously it has been thought that *Tribolium* adult legs come directly from larval leg cells and tissues. In contrast, in more derived Holometabolous insects, such as the tobacco hornworm, *Manduca sexta*, larval legs have set aside cells called imaginal cells that eventually contribute to much of the adult legs. Our findings contradict the theory that *Tribolium* larval legs transform directly into adult legs and in fact suggest that *Tribolium* leg

transformations occur as they do in other insects like *Manduca* (Tanaka and Truman, 2005). In unablated larvae, removal of Hh does not prevent the formation of legs and allows development of long albeit severely distorted pupal legs (Fig. 2 O-R). In contrast, ablated legs were unable to create pupal legs, indicating that Hh is necessary to either set aside cells that are required to create the pupal legs or to maintain proliferation of these set-side cells. Regardless of the actual function, whatever cells are required to recreate the pupal legs must be re-established relatively late during the regeneration process.

Implications of these findings lead to the reevaluation of imaginal disc evolution in insects. Currently, imaginal discs are thought to have evolved only in higher insects, such as *Manduca* and *Drosophila*. Therefore, hemimetabolous insects and holometabolous insects that branched off earlier, such as *Tribolium*, have traditionally been thought to incorporate simpler mechanisms of adult leg development though the direct transformation of nymphal or larval tissues into adult tissues, bypassing the need for imaginal discs. Hence, if *Tribolium* do use imaginal cells during larval to adult leg transformation, this finding implies an intriguing hypothesis that metamorphosis and imaginal cells may have evolved concomitantly and in fact, the origin of imaginal cells may have facilitated the evolution of metamorphosis.

Concluding remarks and speculations

Overall, this study emphasizes the role that Hh plays as a regulator for limb regeneration, growth, proliferation, and possibly for the production of imaginal cells. Together, these findings suggest that Hh might be involved in the maintenance and/or proliferation of stem-cell like cells. Previously, studies have shown that the Hh signaling pathway represents a highly conserved mechanism for maintaining stem cells in many niche systems (Martinez-Agosto *et al*., 2007). For

example, Hh signaling is required for self-renewal and maintenance of the *Drosophila* ovary somatic stem cell (Zhang and Kalderon 2001), the mouse intestinal epithelium (Ramalho-Santos *et al*. 2000), neural stem cells (Lai *et al*. 2003), and hair follicle niches (Gritli-Linde *et al*. 2007). Thus, Hh signaling may be a universal regulator for the maintenance and proliferation of stem cells throughout the Metazoa.

REFERENCES

Alcedo, J. *et al*., 1996. The *Drosophila* Smoothened Gene Encodes a Seven-Pass Membrane Protein, a Putative Receptor for the Hedgehog Signal. Cell. 86, 221-232.

Aza-Blanc, P. *et al*., 1997. Proteolysis that is Inhibited by Hedgehog Targets Cubitus Interruptus Protein to the Nucleus and Converts it to a Repressor. Cell. 89, 1043-1053.

Baker, N. E., 1988. Embryonic and Imaginal Requirements for Wingless, a Segment-Polarity Gene in *Drosophila*. Developmental Biology. 125, 96-108.

Basler, K. & Struhl, G., 1994. Compartment Boundaries and the Control of *Drosophila* Limb Pattern by Hedgehog Protein. Nature. 368, 208-214.

Beermann, A. *et al*., 2001. The Short Antennae Gene of *Tribolium* is Required for Limb Development and Encodes the Orthologue of the *Drosophila* Distal-Less Protein. Development. 128, 287-297.

Bely, A. E. & Nyberg, K. G., 2009. Evolution of Animal Regeneration: Re-Emergence of a Field. Trends in Ecology and Evolution. 25, 161-168.

Blair, S. S., 1995. Compartments and Appendage Development in *Drosophila*. Bioessays. 17, 299-309.

Borod, E. R. & Heberlein, U., 1998. Mutual Regulation of Decapentaplegic and Hedgehog During the Initiation of Differentiation in the *Drosophila* Retina. Developmental Biology. 197, 187-197.

Brockes, J. P., 1997. Amphibian Limb Regeneration: Rebuilding a Complex Structure. Science. 276, 81-86.

Campbell, G. *et al*., 1993. Axis Specification in the Developing *Drosophila* Appendage: The Role of Wingless, Decapentaplegic, and the Homeobox Gene Aristaless. Cell. 74, 1113-1123.

Campbell, G., & Tomlinson, A., 1995. Initiation of the Proximodistal Axis in Insect Legs. Development. 121, 619-628.

Carlson, B. M., 2007. Principles of Regenerative Biology. Elsevier, Ann Arbor, Michigan.

Chen, Y. & Struhl, G., 1996. Dual Roles for Patched in Sequestering and Transducing Hedgehog. Cell. 87, 553-563.

Couso, J. P. *et al*., 1993. A Wingless-Dependent Polar Coordinate System in *Drosophila* Imaginal Discs. Science. 259, 484-489.

Das, S. & Durica, D. S., 2013. Ecdysteroid Receptor Signaling Disruption Obstructs Blastemal Cell Proliferation During Limb Regeneration in the Fiddler Crab, *Uca Pugilator*. Molecular and Cellular Endocrinology. 365, 249-259.

Diaz-Benjumea, F. J. *et al*., 1994. Cell Interaction Between Compartments Established the Proximal-Distal Axis of *Drosophila* Legs. Nature. 372, 175-179.

Dominguez, M. *et al*., 1996. Sending and Receiving the Hedgehog Signal: Control by the *Drosophila* Gli Protein Cubitus Interruptus. Science. 272, 1621-1625.

Duboule, D. & Dollé, P., 1989. The Structural and Functional Organization of the Murine HOX Gene Family Resembles that of *Drosophila* Homeotic Genes. The EMBO Journal. 8, 1497-1505.

Endo, T. *et al*., 2004. A Stepwise Model System for Limb Regeneration. Developmental Biology. 270, 135-145.

Feinberg, E. H., & Hunter, C. P., 2003. Transport of Dsrna into Cells by the Transmembrane Protein SID-1. Science. 301, 1545-1547.

Fire, A. *et al*., 1998. Potent and Specific Genetic Interference by Double-Stranded RNA in *Caenorhabditis Elegans.* Nature. 391, 806-811.

Fortunato, A., & Fraser, A. G., 2005. Uncover Genetic Interactions in *Caenorhabditis Elegans* by RNA Interference. Bioscience Reports. 25, 299-307.

French, V., 1982. Leg Regeneration in Insects: Cell Interactions and Lineage. Journal of American Zoology. 22, 79-90.

Gaunt, S.J. *et al*., 1988. Spatially Restricted Domains of Homeo-Gene Transcripts in Mouse Embryos: Relation to a Segmented Body Plan. Development. 104, 169-179.

Graham, A. *et al*., 1989. The Murine and *Drosophila* Homeobox Clusters Have Common Features of Organization and Expression. Cell. 57, 367-378.

Gritli-Linde, A., *et al*., 2007. Abnormal Hair Development and Apparent Follicular Transformation to Mammary Gland in the Absence of Hedgehog Signaling. Developmental Cell 12, 99–112.

Gunamalai, V. *et al*., 2004. Hormonal Coordination of Molting and Female Reproduction by Ecdysteroids in the Mole Crab *Emerita Asiatica* (Milne Edwards). General and Comparable Endocrinology. 138, 128-138.

Held, L. I. *et al*., 1994. Interactions of Decapentaplegic, Wingless and Distal-Less in the *Drosophila* Leg. Roux's Archive of Developmental Biology. 203, 310-319.

Hopkins, P. M. *et al*., 1999. Limb Regeneration in the Fiddler Crab, *Uca Pugilator*: Histological, Physiological and Molecular Considerations. American Journal of Zoology. 39, 513-526.

Hughes, C. & Kaufman, T. C., 2000. RNAi Analysis of Deformed, Proboscipedia and Sex Combs Reduced in the Milkweed Bug *Oncopeltus Fasciatus*: Novel Roles for Hox Genes in the Hemipteran Head. *Development*, 127: 3683-3694.

Imokawa, Y. & Yoshizato, K., 1997. Expression of Sonic Hedgehog Gene in Regenerating Newt Limb Blastemas Recapitulates that in Developing Limb Buds. *Proceedings of the National Academy of Sciences*. 94, 9159–9164.

Ingham, P. W., 1995. Signaling by Hedgehog Family Proteins in *Drosophila* and Vertebrate Development. Current Opinion in Genetics and Development. 5, 492-498.

Ingham, P. W. & Fietz, M. J., 1995. Quantitative Effects of *Hedgehog* and *Decapentaplegic* Activity on the Patterning of the *Drosophila* Wing. Current Biology. 5, 432-440.

Konopova, B. & Jindra, M., 2007. Juvenile Hormone Resistance Gene Methoprene-Tolerant Controls Entry into Metamorphosis in the Beetle *Tribolium Castaneum*. Proceedings of the National Academy of Sciences. 104, 10488-10493.

Kragl, M. *et al*., 2009. Cells Keep a Memory of Their Tissue Origin During Axolotl Lim Regeneration. Nature. 460, 60-65.

Lai, K., *et al*., 2003. Sonic Hedgehog Regulates Adult Neural Progenitor Proliferation In Vitro and In Vivo. Nature Neuroscience. 6, 21–27.

Marsh, J. L. & Theisen, H., 1999. Regeneration in Insects. Cell and Developmental Biology. 10, 365-375.

Martinez-Agosto, J. A. *et al*., 2007. The Hematopoietic Stem Cell and its Niche: A Comparative View. Genes and Development. 21, 3044-3060.

Mitten, E. K. *et al*., 2012. Matrix Metalloproteinases (MMPs) are Required for Wound Closure and Healing During Larval Leg Regeneration in the Flour Beetle, *Tribolium Castaneum*. Insect Biochemistry and Molecular Biology. 42, 854-864.

Nacu, E. & Tanaka, E. M., 2011. Limb Regeneration: A New Development? Annual Review of Cell and Developmental Biology. 27, 409-440.

Nakamura, T. *et al*., 2008. Dissecting Insect Leg Regeneration through RNA Interference. Journal of Cell and Molecular Life Sciences. 65, 64-72.

Nakano, Y. *Et al*., 1989. A Protein With Several Possible Membrane-Spanning Domains Encoded By The *Drosophila* Segment Polarity Gene Patched. Nature. 341, 508-513.

Ohlmeyer, J. T. & Kalderon, D., 1998. Hedgehog Stimulates Maturation of Cubitus Interruptus Into a Labile Transcriptional Activator. Nature. 396, 749-753.

Pappu, K. S., *et al*., 2005. Mechanism of *Hedgehog* Signaling During *Drosophila* Eye Development. Development. 130, 3053-3062.

Park, T., 1934. Observations on the General Biology of the Flour Beetle, *Tribolium Confusum*. Quarterly Review of Biology. 9, 36-54.

Raftery, L. A., *et al*., 1991. The Relationship of Decapentaplegic and Engrailed Expression in *Drosophila* Imaginal Disks: Do These Genes Mark the Anteriorposterior Compartment Boundary? Development. 113, 27-33

Ramalho-Santos, M. *et al*. 2000. Hedgehog Signals Regulate Multiple Aspects of Gastrointestinal Development. Development. 127, 2763–2772.

Sanicola, M., *et al*., 1995. Drawing a Stripe in *Drosophila* Imaginal Disks: Negative Regulation of Decapentaplegic and Patched Expression by Engrailed. Genetics. 139, 745-756.

Sehnal, F., *et al*., 1996. From Metamorphosis: Postembryonic Reprogramming of Gene Expression on Amphibian and Insect Cells, eds Gilbert LI, Tata JR, Atkinson BG (Academic, San Diego), pp 3–58.

Seifert, A. W., *et al*., 2012. The Influence of Fundamental Traits on Mechanisms Controlling Appendage Regeneration. Biological Reviews. 87, 330-345.

Shah, M. V. *et al*., 2011. The Role of Canonical Wnt Signaling in Leg Regeneration and Metamorphosis in the Red Flour Beetle, *Tribolium Castaneum*. Mechanisms of Development. 128, 342-358.

Simmer, F., *et al*., 2002. Loss of the Putative RNA-Directed RNA Polymerase RRF-3 Makes *C. Elegans* Hypersensitive to RNAi. Current Biology. 12, 1317-1319.

Simon, H. & Tabin C. J., 1993. Analysis of Hox-4.5 and Hox-3.6 Expression During Newt Limb Regeneration: Differential Regulation of Paralogous Hox Genes Suggest Different Roles for Members of Different Hox Clusters. Development. 117, 1397-1407.

Singh, B. N. *et al*., 2012. Hegdehog and Wnt Coordinate Signaling in Myogenic Progenitors and Regulate Limb Regeneration. Developmental Biology. 371, 23-34.

Stark, D. R. *et al*., 1998. Hedgehog Family Member is Expressed Throughout Regenerating and Developing Limbs. Developmental Dynamics. 212, 352-363.

Struhl, G. & Basler, K., 1993. Organizing Activity of Wingless Protein in *Drosophila*. Cell. 75, 527-540.

Suzuki, Y. *et al*., 2009. Larval Leg Integrity is Maintained by Distal-Less and is Required for Proper Timing of Metamorphosis in the Flour Beetle, *Tribolium Castaneum*. Developmental Biology. 326, 60-67.

Tanaka, E. M., 2003. Regeneration: If They Can Do It, Why Can't We? Cell. 113, 559-562.

Tribolium Genome Sequencing Consortium, 2008. The Genome of the Model Beetle and Pest *Tribolium Castaneum*. Nature. 452, 949–955.

Tomoyasu, Y. & Denell, R.E., 2004. Larval RNAi in *Tribolium* (Coleoptera) for Analyzing Adult Development. Development Genes and Evolution. 214, 575-578.

Trowbridge, J. J. *et al*., 2006. Hedgehog Modulates Cell Cycle Regulators in Stem Cells to Control Hematopoietic Regeneration. PNAS. 103, 14134-14139.

Truby, P. R., 1983. Blastema Formation and Cell Division during Cockroach Limb Regeneration. Journal of Embryology and Experimental Morphology. 75, 151-164.

Truman, J. W. & Riddiford, L. M., 1999. The Origins of Insect Metamorphosis. Nature. 401, 447-452.

Truman, J. W., 2005. Hormonal Control of Insect Ecdysis: Endocrine Cascades for Coordinating Behavior with Physiology. Vitamins and Hormones. 73, 1-30.

Winston, W. M., *et al*., 2002. Systemic RNAi in *C. Elegans* Requires the Putative Transmembrane Proein SID-1. Science. 295, 2456-2459.

Wolpert, L., 1969. Positional Information and the Spatial Pattern of Cellular Differentiation. Journal of Theoretical Biology. 25, 1–47.

Zhang, Y. & Kalderon, D., 2001. Hedgehog Acts as a Somatic Stem Cell Factor in the *Drosophila* Ovary. Nature. 410, 599–604.

Zecca, M. *et al*., 1995. Sequential Organizing Activities of Engrailed, Hedgehog and Decapentaplegic in the *Drosophila* Wing. Development. 121, 2265-2278.